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(54) Title: VACCINE CONTAINING A PEROXIREDOXIN AND/OR A β -TUBULIN (57) Abstract <p>The present application relates to vaccine compositions comprising peroxiredoxin and/or β-tubulin antigenic material, preferably of <i>Fasciola</i> or <i>Dicrocoelium</i> origin, for use in combatting a parasitic infestation of helminths in a mammal. It further relates to nucleic acid sequences which encode peroxiredoxin and/or β-tubulin molecules and the amino-acid sequences thereof, vectors comprising said nucleic acid sequences and cells transformed with such vectors.</p>		

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VACCINE CONTAINING A PEROXIREDOXIN AND/OR A β -TUBULIN

- 5 The invention relates to the use of a peroxiredoxin (thiol-specific antioxidant) and/or a β -tubulin as a protective antigen against helminth parasites.

10 Each species of domestic animal can be parasitised by a number of different species of helminth, a process which usually causes disease. For example, the parasitic trematode Fasciola hepatica is known to be one cause of the economically important disease fascioliasis in ruminants, such as cattle and sheep. The parasite
15 enters the mammalian host by penetrating the gut wall and spends approximately seven weeks feeding on and burrowing through the liver mass before migrating into the bile duct. Following infection, development of immunity in the host can be poor and resistance to
20 reinfection in already infected hosts may be only partial or non-existent. Other parasitic flukes include Fasciola gigantica and Dicrocoelium spp., Paramphistomum spp. and also Schistosoma spp., eg S.bovis and S.mansoni.

25 Problems are also caused by nematodes such as hookworms (e.g. Necator, Ancylostoma, Uncinaria and Bunostomum spp.).

30 Of the blood feeding nematodes the genus Haemonchus causes anaemia and weight loss and if untreated frequently leads to death. Animals infected with the related non-blood feeding nematode Ostertagia similarly fail to thrive and may die if untreated.

35 Other parasitic worms of economic importance include the various species of the following helminth genera:-

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Trichostrongylus, Nematodirus, Dictyocaulus, Cooperia,
Ascaris, Dirofilaria, Trichuris and Strongylus. In
addition to domestic livestock, companion animals and
humans may also be infected, not infrequently with fatal
5 results and helminth infections and infestations thus
pose a problem of considerable worldwide significance.

Control of helminth parasites of grazing livestock
currently relies primarily on the use of anthelmintic
10 drugs combined with pasture management. Such techniques
are often unsatisfactory, firstly because anthelmintic
drugs may have to be administered frequently, secondly
because resistance against anthelmintic drugs is
becoming increasingly widespread and thirdly because
15 appropriate pasture management is often not possible on
some farms and even where it is, it can place
constraints on the best use of available grazing.

Numerous attempts have been made to control helminth
20 parasites of domestic animals by immunological means.
With very few exceptions (e.g. the cattle lungworm,
Dictyocaulus viviparus) this has not proved possible.

A vaccine against F.hepatica has been proposed in
25 W090/08819 comprising a glutathione-S-transferase from
F.hepatica as antigenic material. Further vaccines
against F.hepatica have been proposed in W094/09142,
W094/28925 and PCT/GB95/02350 comprising respectively a
Cathepsin L, a dipeptidyl peptidase and a class of
30 haemoproteins from F.hepatica as antigenic material.

Bennett (UK Patent No. 2169606B) extracted various
antigens from Fasciola organisms by a process which
separates antigens specific to the juvenile stage from
35 antigens present throughout the juvenile and adult
stages.

Furthermore crude in vitro excretory/secretory (E/S) products can under some circumstances confer immunity on rats (Rajasekariah et al, Parasitol. 79 (1979), p. 393-400).

5

It has now been found that animals vaccinated against F.hepatica using a relatively impure haemoprotein preparation, the pure counterpart of which is described in PCT/GB95/02350, produce antibodies against

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peroxiredoxin and β -tubulin molecules of fluke origin.

This discovery opens up the possibility of vaccines against F.hepatica and other helminths based on the use of peroxiredoxin and/or β -tubulin molecules and/or corresponding proteins produced by other helminth

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parasites as antigens.

Accordingly an aspect of the present invention provides a vaccine composition for use in combating a parasitic infestation of helminths in a mammal wherein the

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antigenic material comprises a peroxiredoxin and/or a β -tubulin molecule, in at least partially purified form, or an antigenic fragment or epitope, component, precursor, analogue, variant or functionally equivalent derivative thereof, together with a carrier and/or

25

adjuvant.

The invention also provides a method of combating a parasitic infestation of helminths in a mammal comprising administering to said mammal a vaccine

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according to the invention as hereinbefore defined in an amount effective to combat said infestation.

Alternatively viewed, the invention provides for the use of the molecules as hereinbefore described in the preparation of a vaccine composition for combatting a

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parasitic infestation of helminths in a mammal.

The mammal is preferably a ruminant, for example cattle or sheep, but the vaccine and method of the invention may also find application in humans, companion animals such as dogs and cats or in other domestic animals.

5

Preferably the peroxiredoxin and/or β -tubulin molecules are derived from flukes such as Fasciola or Dicrocoelium, in particular from the liver fluke Fasciola hepatica. Alternatively it is preferred that the peroxiredoxin and/or β -tubulin molecules should be capable of stimulating an immune response which will be effective against Fasciola or Dicrocoelium, in particular F. hepatica and F. gigantica, such peroxiredoxin and/or β -tubulin molecules from other species as are capable of conferring a cross-protective immune response thus forming a particularly preferred aspect of the invention.

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The F. hepatica peroxiredoxin and β -tubulin molecules shown hereinafter to possess cDNA sequences and predicted amino acid sequences as shown in Figs. 2 and 4 respectively are particularly preferred for use in the vaccine and method of the invention.

20

The peroxiredoxin and/or β -tubulin molecules incorporated in the vaccine according to the invention are in at least partially purified form. Preferably the molecules of the present invention are at least 75% pure and more preferably at least 95% pure. It will be appreciated that once peroxiredoxin and/or β -tubulin molecules of at least 95% purity have been obtained they can be admixed with one or more further purified antigenic proteins, to form a polyvalent vaccine.

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According to the present invention the peroxiredoxin and/or β -tubulin molecules incorporated in the vaccine may be in the form of antigens, antigenic fragments,

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- 5 -

epitopes, components, precursors, analogues or functionally-equivalent derivatives thereof.

5 A preferred form of polyvalent vaccine according to the invention will contain peroxiredoxin and/or β -tubulin polypeptides as referred to above in combination with a Cathepsin L-type antigen as described in more detail in International Patent Application No. WO94/09142 or a dipeptidyl peptidase antigen as described in more detail
10 in International Patent Application No. WO94/28925 or a class of haemoprotein molecules as described in more detail in International Patent Application No. PCT/GB95/02350. Thus the Cathepsin L and/or dipeptidyl peptidase and/or haemoproteins are preferably derived
15 from flukes such as Fasciola or Dicrocoelium, in particular the liver fluke F.hepatica. Such a polyvalent vaccine will, by inducing immunity in the host species against two or more separate aspects of the invading helminth parasite, significantly increase the
20 likelihood of protection against the helminth and significantly reduce the chances of infestation occurring.

25 Monovalent vaccines according to the invention may also have an anti-fecundity effect on helminth parasites, and this effect should be still more marked with polyvalent vaccines.

30 In a preferred aspect the polyvalent vaccine comprises peroxiredoxin and/or β -tubulin polypeptides according to the present invention together with a Cathepsin L1 having molecular weight of 27 kDa by sodium dodecyl sulphate polyacrylamide gel electrophoresis as disclosed in WO94/09142 and/or a Cathepsin L2 having molecular
35 weight of 29.5 kDa by the same technique as disclosed in WO94/09142 and/or a dipeptidyl peptidase having molecular weight of 200 kDa by the same technique as

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disclosed in WO94/28925 or one or more of a class of haemoproteins of at least 200 kDa by gel filtration chromatography as disclosed in PCT/GB95/02350.

- 5 The vaccines according to the invention may be formulated with conventional carriers and/or adjuvants and the invention also provides a process for the preparation of the vaccines comprising bringing into association purified peroxiredoxin and/or β -tubulin
- 10 molecules as hereinbefore described and one or more adjuvants or carriers. Suitable adjuvants include aluminium hydroxide, saponin (ISCOMs), quil A and more purified forms thereof, muramyl dipeptide, mineral and vegetable oils, DEAE dextran, nonionic block copolymers
- 15 or liposomes such as Novasomes (Trade Mark of Micro Vesicular Systems Inc.), in the presence of one or more pharmaceutically acceptable carriers or diluents. Carriers for peptide sequences corresponding to epitopes of peroxiredoxin or β -tubulin molecules according to the
- 20 invention can be proteins such as Hepatitis B core antigen multiple antigen peptide or lipopeptides such as tripalmitoyl-S-glycerylcysteinylserine (P₃CSS). Suitable diluents include liquid media such as saline solution appropriate for use as vehicles. Additional
- 25 components such as preservatives may be included.

- Administration of the vaccine to the host species may be achieved by any of the conventional routes, e.g. orally or parenterally such as by intramuscular injection,
- 30 optionally at intervals e.g. two injections at a 7-35 day interval. A suitable dose when administered by injection might be such as to give an amount of protein within the range 10-500 μ g.

- 35 According to a further aspect, the invention provides the F.hepatica peroxiredoxin molecule or antigenic fragments, epitopes, components, precursors, analogues

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or variants thereof and functionally-equivalent derivatives thereof having protective antigenic activity against one or more helminth parasites, characterised by:

- 5
- (a) having at least a portion which substantially corresponds to the amino acid sequence as shown in Fig. 4;
- 10 (b) being encoded by a nucleotide sequence at least a portion of which substantially corresponds to the sequence shown in Fig. 4;

While the peroxiredoxin and/or β -tubulin molecules for use in the vaccine according to the invention may be prepared by isolation from the helminths, it may also be convenient to prepare them by recombinant DNA techniques with the known advantages which such techniques give in terms of scaling-up of production and reproducibility.

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20 Thus the invention also provides for peroxiredoxin and β -tubulin molecules as hereinbefore described, produced by means of recombinant DNA techniques.

Accordingly, in one aspect, the present invention provides for nucleic acid sequences which encode the peroxiredoxin or the β -tubulin molecules of the invention or antigenic portions thereof substantially corresponding to all or a portion of the nucleotide sequences as shown in Fig. 4 for peroxiredoxin and Fig. 2 for β -tubulin or sequences encoding helminth peroxiredoxin or β -tubulin antigens which are substantially homologous or which hybridise with any of said sequences.

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35 A nucleic acid according to the invention may thus be single or double stranded DNA, cDNA or RNA.

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Variations in the peroxiredoxin or β -tubulin-encoding nucleotide sequences may occur between different strains of helminth within a species, between different stages of a helminth life cycle (e.g. between larval and adult stages), between similar strains of different geographical origin, and also within the same helminth. Such variations are included within the scope of this invention.

"Substantially homologous" as used herein includes those sequences having a sequence identity of approximately 50% or more, eg. 60% or more, and also functionally-equivalent allelic variants and related sequences modified by single or multiple base substitution, addition and/or deletion. By "functionally equivalent" is meant nucleic acid sequences which encode polypeptides having anti-oxidant or β -tubulin functionality which are similarly immunoreactive i.e. which raise host protective antibodies against helminths.

Nucleic acid molecules which hybridise with the sequences shown in Figs. 2 and 4 or any substantially homologous or functionally equivalent sequences as defined above are also included within the scope of the invention. "Hybridisation" as used herein defines those sequences binding under non-stringent conditions (6 x SSC/50% formamide at room temperature) and washed under conditions of low stringency (2 x SSC, room temperature, more preferably 2 x SSC, 42°C) or conditions of higher stringency eg. 2 x SSC, 65°C (where SSC = 0.15M NaCl, 0.015M sodium citrate, pH 7.2).

Methods for producing such derivative related sequences, for example by site-directed mutagenesis, random mutagenesis, or enzymatic cleavage and/or ligation of

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nucleic acids are well known in the art, as are methods for determining whether the thus-modified nucleic acid has significant homology to the subject sequence, for example by hybridisation.

5

Provision of a nucleic acid molecule according to the invention thus enables recombinant peroxiredoxin or β -tubulin or immunogenic fragments thereof, to be obtained in quantities heretofore unavailable, thereby permitting the development of anti-helminth vaccines.

10

In another aspect the present invention thus provides nucleic acid molecules comprising one or more nucleotide sequences encoding one or more polypeptides capable of raising protective antibodies against helminth parasites, which sequences incorporate one or more antigenic determinant-encoding regions from the peroxiredoxin or β -tubulin encoding sequences as shown in Figs. 2 and 4.

15

20

The present invention also extends to synthetic polypeptides comprising one or more amino acid sequences constituting a peroxiredoxin or β -tubulin molecule or antigenic portions thereof, substantially corresponding to all or a portion of the nucleotide sequences as shown in Figs. 2 and 4 or a functionally-equivalent variant thereof.

25

Additional aspects of the invention related to the above include vectors containing one or more nucleotide sequences as defined above; host cells, for example bacteria such as E. coli or yeast cells such as Saccharomyces spp., or more preferably eukaryotic cells, transformed by such vectors, for example by a baculovirus vector; and processes for preparing recombinant peroxiredoxin and β -tubulin polypeptides or antigenic fragments or epitopes thereof comprising

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culturing such transformed host cells and isolating said peroxiredoxin or β -tubulin polypeptides or fragments or epitopes from the cultured cells.

- 5 An alternative live or inactivated vaccine formulation may comprise an attenuated or virulent virus or a host cell, e.g. a microorganism such as a bacterium, having inserted therein a nucleic acid molecule (e.g. a DNA molecule) according to the invention for stimulation of
10 an immune response directed against polypeptides encoded by the inserted nucleic acid molecule. A bacterial vector which elicits local gut mucosal immunity to a fluke antigen which then blocks juvenile fluke migration is particularly preferred, notably invasive species such
15 as Salmonella species.

- Additional antigenic materials may also be present in the vaccine thus giving an enhanced protective effect against the helminth parasite in question or a combined
20 protective effect against one or more additional parasitic infestations.

- A yet further aspect of the invention provides monoclonal or polyclonal antibodies capable of inducing
25 immunity to peroxiredoxin or β -tubulin molecules in a mammal when administered to said mammal, the antibodies having an affinity for the variable region of one or more further antibodies, said further antibodies having an affinity for said thiol-specific antioxidant or β -
30 tubulin molecules.

- This approach, the so-called "anti-idiotypic" approach, permits formulation of a vaccine which will dispense entirely with the original antigen and may offer even
35 greater advantages in terms of safety, avoidance of side effects and convenience of manufacture.

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Brief Description of the DrawingsFigure 1

PCR amplified inserts of immunoselected λ gt11 clones.

- 5 Positive clones were amplified by PCR using universal λ forward and reverse primers. Samples of each PCR reaction were analysed by agarose gel electrophoresis.

lanes 1,12,23

10 pGem DNA markers

lane 2	clone	D6
lanes 3 & 4	clones	B5, D5
lanes 5-11	clones	A1, A4, A5, B1, B4, B6, E3
lane 13	clone	C4
15 lanes 14-17	clone	C2, D1, D7, E2
lane 18	clone	D8
lanes 19&20	clones	C1, D3
lane 21	clone	A8
lane 22	clone	E4

20

Figure 2

Nucleotide sequence and deduced amino acid sequence of β -tubulin (clone D6).

25 Figure 3

Alignment of predicted amino acid sequence of clone D6 to Toxoplasma β -tubulin.

The deduced amino acid sequence of the partial D6 sequence was aligned with that of β -tubulin from

- 30 Toxoplasma gondii (GenBank accession no. P10878, Nagel and Boothroyd, 1988). Boxes surround homologous regions and gaps have been introduced to give maximum alignment. Z = not determined.

35 Figure 4

Nucleotide sequence and predicted amino acid sequence of (peroxiredoxin) (clone B1).

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Figure 5

Alignment of predicted amino acid sequence of clone B1. The deduced amino acid sequence of clone B1 was aligned with that of rat thiol-specific antioxidant (TSA,

- 5 GenBank accession no. P35704), human natural killer cell enhancing factor B, (NKEF B, accession no. P31945), human proliferation associated gene, (PAG, accession no. X67951), human TSA (Lim et al, 1994, accession no. P35701), and Onchocerca volvulus TSA (accession no. 10 U09385). Boxes denote conserved residues and gaps have been introduced to maximise alignment. The active site cysteine residues are indicated by arrows.

Figure 6

- 15 Expression of clone B1 fusion protein.

A. Plate wash supernatants of wild type phage (lane 1) and clone B phage (lane 2) were subjected to reducing SDS PAGE and silver staining.

20

B. Following electrophoresis, SDS gels were blotted onto nitrocellulose and probed with anti- β -galactosidase antibody. Lane 1 contains wild type phage supernatant and lane 2 contains clone B1 supernatant. Large arrows 25 indicate the position of β -galactosidase. Small arrows indicate the position of B1 recombinant fusion protein.

Figure 7

- 30 Northern blot analysis of total RNA from F.hepatica and bovine liver.

Total RNA from F.hepatica (lane 1) and from bovine liver (lane 2) was electrophoresed in a formaldehyde agarose gel, transferred to a nitrocellulose filter and probed with 32 P labelled 400bp fragment. RNA size markers are 35 indicated.

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Figure 8

Protection of glutamine synthetase by liver fluke homogenate against the DTT/Fe³⁺ system.

0.5 U glutamine synthetase (GS) was incubated in the presence of the inactivating solution (IS): 15 μ M FeCl₃, and 5 mM DTT, with 0.3 mg, 0.6 mg and 0.9 mg liver fluke homogenate (LFH), for 10 min at 37°C. Reactions were then assayed for remaining glutamine synthetase activity.

Detailed Description of the Invention:1. Materials

The Alpha ³²P dATP was obtained from Amersham, the RNazol™ B from AMS Biotechnology Ltd., and the X-Omat X-ray film, FX 40 liquid Fixer, LX 24 developer 667 Polaroid film were all purchased from Kodak. Agarose, Anti- β galactosidase antibody labelled with alkaline phosphatase (mouse), Apa I, 5-bromo-4-chloro-3-indolyl- β -D-galactosidase (X-Gal), dNTP's, EcoR I, Hind III, isopropylthio- β -D-galactoside (IPTG), pGem DNA markers, pGem® vector system, Prime-a-Gene® system, Sac I, Taq DNA Polymerase, Wizard™ λ preps, Wizard™ DNA clean-up system were all purchased from Promega. Adenine diphosphate (ADP), anti-bovine IgG conjugated to alkaline phosphatase (rabbit), diethylpyrocarbonate (DEPC), dithiothreitol (DTT), glutamine, glutamine synthetase, lysozyme, proteinase K, salmon sperm DNA all came from Sigma Chemical Company.

2. Immunoscreening of F hepatica λ gt11 cDNA expression library35 Preparation of λ gt11 cDNA library

A λ gt11 cDNA library was prepared by the following

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standard method (Promega Handbook). Total RNA was isolated from mature adult flukes using RNazoL™. From this, mRNA was isolated by binding to an oligo dT column. Double stranded cDNA was generated from the mRNA using the Riboclone® cDNA synthesis kit. EcoR I linker arms were added to the cDNA, which was then ligated to gt11 arms and packaged into λ heads using the Packagene® system. The packaged phage was titred and then amplified by infecting phage competent E. coli Y1090 cells (overnight culture grown in LB media with 0.2% maltose and 10 mM MgSO₄) with dilutions of the phage, incubating at room temperature for 20 min and then plating the bacteria in top agar onto LB agar plates with 100 μ g ml⁻¹ ampicillin.

15

Preparation of haemoglobin fraction

Mature F. hepatica flukes were removed from the bile ducts of infected livers from condemned cattle at a local abattoir in Ireland. The flukes were washed six times in phosphate buffered saline (PBS), pH 7.3, and then maintained in RPMI-1640, pH 7.3, containing 2% glucose, 30 mM HEPES and 25 mg ml⁻¹ gentamycin at 37°C for 18 hours. Following this incubation period the culture medium was removed, centrifuged at 12,00 x g for 30 minutes and the supernatant (ES products) collected and stored at -20°C.

Five hundred ml of ES products were concentrated to 15 ml in an Amicon 8400 Ultrafiltration unit (Danvers, MA, USA) with a YM3 membrane (3,000 mw cut-off). The concentrated sample was centrifuged at 12,000 x g for 30 minutes and applied to a 340 ml Sephacryl S-200 column equilibrated in 0.1M Tris-HCl, pH 7.0, at 4°C. Fractions (5 ml) were collected after the void volume (110 ml) had been passed. The absorbance of the eluate was monitored at 280 nm using an Atto UV Monitor. Those

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fractions containing haemoprotein (yellow coloured) were pooled and concentrated in an Amicon 8050 Ultrafiltration unit to 5 ml. This concentrate was termed haemoglobin fraction (Hf).

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Preparation of sera for immunoscreening

The cDNA library was immunologically screened using a pool of sera from animals vaccinated with haemoglobin fraction (Hf) as described above. The sera were obtained following three vaccinations with Hf and prior to parasite challenge. Before use the sera was pre-adsorbed to remove all antibodies reactive with E. coli proteins. This was achieved by incubating the sera with nitrocellulose discs containing bound E. coli proteins at room temperature for 6 h. This adsorption procedure was repeated three times. The discs were prepared by incubating the discs in a sonicated extract of E. coli cells (10 x 30 sec bursts, duty cycle 0.7 sec) for 24 h at 4°C and then blocking the excess sites with 1% BSA/ T-PBS. Sera was incubated with discs, removed, centrifuged and stored at 4°C until required.

Immunoscreening of λ library

25

Phage competent E. coli Y1090 were infected with 1:50 dilution of phage. Following an incubation for 20 min at room temperature the cells were plated in top agar on LB ampicillin plates and incubated at 42°C until plaques were visible (ca 3 h). Nitrocellulose discs which had been soaked in 10 mM IPTG and air dried, were carefully placed on the plates and their orientation was marked by three needle stabs. The plates were incubated for 4 h at 37°C, the discs were then carefully removed and blocked overnight in 1% BSA/T-PBS, before probing with the pre-adsorbed bovine antisera (1:500 dilution). Following washing in T-PBS bound antibody was detected

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using alkaline phosphatase labelled anti-bovine IgG, with NBT and BCIP as substrate. Positive plaques appeared as purple rings. These plaques were removed as an agar plug using a sterile pasteur pipette, transferred to 1 ml phage buffer (10 mM MgSO₄, 100 mM NaCl, 20 mM Tris-HCl, pH 7.4) and allowed to diffuse at 4°C overnight. Individual phage were re-plated and the antibody screening repeated two additional times or until pure plaques were obtained i.e. when all plaques on a plate were reactive with the antibody.

3. Preparation of λ lysates and isolation of DNA

Isolated plaques were picked into 200 μ l 0.1X SM buffer (0.01% gelatin, 8 mM MgSO₄, 100 mM NaCl, 50 mM Tris-HCl, pH 7.5) and incubated overnight at 4°C. One hundred μ l was used to infect competent Y1090 cells, which were plated as before and incubated at 42°C until confluent lysis was observed (ca 5 h). Four ml 0.1X SM buffer was added to the plate and after an overnight incubation at 4°C the buffer was removed. Chloroform was added (0.5% final concentration) and the lysate was stored at 4°C until required.

4. PCR analysis of λ DNA

Polymerase Chain Reaction (PCR) was employed to isolate and estimate the size of the inserts from the phage library, using universal λ primers. These primers are derived from the sequence flanking the EcoR I cloning site of the λ gt11 vector. Twenty μ l of stock λ lysates was added to 180 μ l water and boiled for 10 minutes and then 1 μ l was used per 50 μ l PCR. Each PCR vial consisted of the following mix:

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	10X Polymerase buffer	5.0 μ l
	dNTP's (1 mM each)	5.0 μ l
	MgCl ₂ (25 mM)	6.0 μ l
	Sterile distilled water	30.7 μ l
5	λ forward primer (50 ng μ l ⁻¹)	1.0 μ l
	λ reverse primer (50 ng μ l ⁻¹)	1.0 μ l
	Taq Polymerase (5U μ l ⁻¹)	0.3 μ l
	λ lysate DNA	1.0 μ l

10 Each mix was overlaid with 70 μ l mineral oil, placed in the Hybaid Omnigene Thermal Cycler, and the PCR carried out as follows:

	Stage 1	(Denaturation)	94°C for 4 min
15	Stage 2	(Denaturation)	94°C for 30 sec
		(Annealing)	55°C for 1 min
		(Extension)	74°C for 1 min 30 sec
	- stage 2 was repeated for 35 cycles		
	Stage 3	(Extension)	74°C for 4 min

20

25 μ l of PCR reactions were analysed by agarose gel electrophoresis as detailed in Sambrook et al (1989).

5. Sub Cloning of PCR fragments

25

PCR amplified gene fragments were excised from the gel. The agarose was disrupted using glass beads, and the recovered DNA was purified using the WizardTM DNA clean-up system (Promega). The fragments were then sub cloned directly into the pGem[®]-T plasmid, as follows:

30

1 μ l (25 ng) pGem[®]-T vector, 8 μ l ligase buffer (10 mM ATP, 100 mM MgCl₂, 100 mM DTT, 300 mM tris-HCl, pH 7.8), 1 U T4 DNA ligase and 100 ng insert DNA were mixed gently and the ligation was allowed to proceed overnight at 4°C.

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Competent cells were prepared using one of the following methods:

(a) calcium chloride transformation

A log phase culture of *E. coli* JM109 cells was
5 aliquoted, placed on ice for 5 min, centrifuged at
12,000 x g for 2 min and the supernatant removed. The
cells were gently resuspended with 1 ml of cold CaCl₂ and
incubated on ice for 30 min. The cells were spun again
and resuspended in 0.5 ml cold CaCl₂. 10 µl ligation mix
10 was carefully added to 50 µl aliquots of cells and
placed on ice for a further 30 min. The cells were then
heat shocked at 42°C for 90 sec and returned to ice for
2-5 min. Immediately after transformation 950 µl pre-
warmed LB media was added and the cells incubated at 37°C
15 for 1 h. Cells were concentrated by centrifugation and
spread on LB plates containing 100 µg ml⁻¹ ampicillin,
0.5 mM IPTG and 40 µg ml⁻¹ X-Gal (for blue/white
selection).

20 (b) electroporation

A log phase culture of *E. coli* XL1-blue electrocompetent
cells was concentrated by centrifugation and aliquoted.
2.5 µl ligation reaction was added to 300 µl cells,
gently mixed and placed in 0.2 µm electroporation
25 cuvettes. The cells were then transformed by
electroporating under the following conditions: the
pulse generator was set at 25 µF, 2.48 kV, and 200 Ω.
One pulse at these settings results in a pulse of 12.5
kV cm⁻¹ with a time constant of ca 4 sec. 1 ml pre-
30 warmed SOC (containing 20 mM glucose) medium was added
immediately and the cells were incubated for 1 h at 37°C,
before concentrating and plating as before. Plates
spread with transformed cells were incubated
overnight at 37°C.

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6. Screening of recombinant plasmids

With X-Gal and IPTG colour screening, recombinant colonies should be white and colonies with no insert DNA blue. White colonies were picked into 2 ml LB with 100 $\mu\text{g ml}^{-1}$ ampicillin (and 15 $\mu\text{g ml}^{-1}$ tetracycline for XL blue cells), and incubated overnight at 37°C. Plasmid DNA from 1 ml of this mini prep culture was isolated by either the boiling or alkali lysis method described by Sambrook et al (1989). The DNA was double digested with the restriction enzymes Sac I and Apa I and the inserts observed on agarose gel electrophoresis.

7. Sequencing of Plasmid DNA

Purified plasmid DNA from positive clones was further cleaned up using Wizard™ λ preps. The DNA was sent for sequence analysis to the Department of Biological Sciences, Durham University or BioResearch Ireland, Trinity College Dublin.

8. Preparation of fusion protein

Sequence analysis revealed that clone B1 was a novel fluke antioxidant protein (peroxiredoxin) which was therefore further characterised. Fusion protein from the λ B1 clone was prepared by the plate wash supernatant method. Phage competent E. coli Y1090 were infected with 10,000 pfu recombinant phage and incubated for 20 min at room temperature, before pouring onto LB ampicillin plates in top agar. The plates were incubated at 42°C for 3 h (lysis almost confluent), then 5 ml phage buffer containing 1 mM EDTA, 1 mM PMSF, 1 mM iodoacetamide and 10 mM IPTG was added to the plates which were incubated at 37°C overnight. The buffer was recovered and the top agar was also scraped into a centrifuge tube. This was vortexed for 20 sec before

- 20 -

centrifuging at 10,000 x g for 10 min at 4°C. The supernatant was removed to microfuge tubes which were spun again at 12,000 x g. Supernatants were stored at -20°C until required.

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The fusion protein was analysed by reducing SDS polyacrylamide gel electrophoresis followed by silver staining, and by immunoblotting using an anti-β galactosidase primary antibody.

10

9. Preparation of radiolabelled DNA probe

A 400 bp fragment was PCR amplified from clone B1 DNA using the following consensus primers, designed from comparing the protein sequences of the peroxiredoxin antioxidant family. These primers crossed the regions that code for the conserved active site regions, cys 47 (VCP 47) and cys 168 (VCP 168).

20

VCP 47 forward primer (Shem F)
5'GAT TTY ACW TTY GTN TGT CCW ACW GAR-3'

VCP 168 reverse primer (SmTSAR)
5' GGW CAN ACY TCW CCA TGY TC -3'

25

where Y= T or C, W= A or G and N= T C, A or G

The PCR product was excised from an agarose gel and cleaned as before. The fragment was labelled with Alpha ³²P by random priming using the Promega Prime-a-Gene® system. The reaction mix was as follows:

30

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	5X labelling buffer	10 μ l
	(250 mM tris-HCl, pH 8.0, 25 mM MgCl ₂ , 10 mM DTT, 1 mM HEPES, pH 6.6, 26 A ₂₆₀ units ml ⁻¹ random hexadeoxyribonucleotides) mixture	
5	of dCTP, dGTP, dTTP (100 mM each)	2 μ l
	acetylated BSA 10 mg ml ⁻¹	2 μ l
	denatured DNA probe	25 ng
	sterile water	25 μ l
	alpha ³² P dATP (50 μ Ci, 3,000 Ci mMol ⁻¹)	5 μ l
10	Klenow enzyme	5 U

The reaction tube was mixed gently and incubated at room temperature for 1 h. 200 μ l 0.5 M EDTA was added and the reaction terminated by boiling for 2 min. The probe was now ready for use in hybridisation reactions.

10. Isolation of RNA and northern blotting

a. Isolation of adult fluke RNA

20 Mature flukes were cultured overnight in RPMI-1640, pH 7.3 containing 2% glucose, 30mM HEPES and 25 mg/l gentamycin, to allow clearing of the gut contents which could contain host cells. Approximately 10 flukes (1 gram tissue) were placed in a centrifuge tube, 5 ml

25 RNAzolTM was added and the flukes were homogenised at top speed for 30 sec using a Thyristor Regler TR50 homogeniser. One ml of chloroform was added and the solution was shaken vigorously for 15 sec and placed on ice for 5 min. After aliquoting into microfuge tubes

30 the solution was centrifuged at 13,000 x g for 15 min at 4°C and two layers formed. The upper aqueous phase was removed to a new tube, an equal volume of isopropanol was added and the samples were incubated at 4°C for 15 min (or aliquoted for long term storage at -80°C). They

35 were recentrifuged for 15 min, the supernatant was removed and the RNA pellet washed with 75% ethanol before drying and reconstitution with 200 μ l 0.1% DEPC

- 22 -

treated water. Bovine RNA was isolated using the same procedure with 1 g fresh bovine liver as starting material. The RNA was analysed by electrophoresis on agarose gels containing formaldehyde as detailed in
5 Sambrook et al (1989):

b. Northern blotting

Following electrophoresis the gel was rinsed with DEPC treated water to remove the formaldehyde and the RNA was
10 transferred onto nitrocellulose membrane by the capillary transfer method outlined by Sambrook et al, (1989). RNA fragments are carried from the gel in a flow of buffer and deposited on the surface of the nitrocellulose. Following transfer, the RNA was fixed
15 onto the membrane by baking for 2 h at 80°C in an oven.

c. Hybridisation with radiolabelled probe

The nitrocellulose filter was soaked in 6X SSC (0.9 M NaCl, 90 mM sodium citrate pH 7.0) until thoroughly
20 wetted and placed in a heat-sealable bag. Then, 200 ml prehybridisation solution (6X SSC, 5X Denhardt's reagent, 0.5% SDS, 100 µg ml⁻¹ denatured, fragmented salmon sperm DNA) was added to the bag. As much air as possible was squeezed from the bag which was sealed and
25 incubated overnight at 68°C. Following incubation the bag was opened by removing a corner and the radiolabelled probe carefully added. The resealed bag was then placed in a second sealed bag and incubated again at 68°C for 24 h. The hybridisation solution was
30 carefully poured into a suitable container and the filters were removed and immediately submerged in 300 ml 2X SSC and 0.1 % SDS. The filters were incubated with gentle agitation at room temperature for 15 min. The wash solution was replaced twice and the incubation
35 repeated. Then 0.1X SSC and 0.5% SDS was added to the filters which were further incubated at 68°C for 1 h. Filters were rinsed with 0.1X SSC to remove the SDS,

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blotted briefly on paper towels and wrapped in clingfilm, and then exposed to X-ray film at -80°C to obtain an autoradiographic image. Exposure for 24 h at 80°C with an intensifying screen was required to obtain an image.

11. Assay of mature fluke extract for novel antioxidant activity

Antioxidant activity in mature liver fluke extract was measured by monitoring its ability to inhibit the thiol/iron/oxygen mediated inactivation of glutamine synthetase. Assays were performed in microtitre plates in a $100\ \mu\text{l}$ reaction volume containing 0.5 U glutamine synthetase (*E. coli*), in the presence or absence of inactivation solutions and protector protein (liver fluke homogenate). Inactivation solutions consisted of $15\ \mu\text{M}$ FeCl_3 and either 5 mM DTT or 14 mM 2-mercaptoethanol (final concentrations). After incubation for 10 min at 37°C remaining glutamine synthetase activity was measured by adding $100\ \mu\text{l}$ of γ glutamyl transferase assay mixture. This contained 0.4 mM ADP, 150 mM glutamine, 10 mM potassium arsenate, 0.4 mM manganese chloride, 20 mM hydroxylammonium chloride in 50 mM imidazole-HCl, pH 7.0. The reaction was incubated at 37°C for 30 min and terminated by the addition of $50\ \mu\text{l}$ stop mixture, consisting of 55 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 20 g trichloroacetic acid and 21 ml concentrated HCl per litre. An absorbance resulting from the γ glutamyl hydroxamate- Fe^{3+} complex was measured at 540 nm. In the absence of "protector protein" under these conditions 70 to 100% of glutamine synthetase activity was lost.

- 24 -

12. Immunoscreening of F.hepatica cDNA library and analysis of isolated clones by PCR and restriction digestion

5 Bovine sera from the vaccine trial was used to screen a F.hepatica cDNA library constructed in λ gt11 phage. The serum pool used was obtained on the day of parasite challenge (week 11) from animals immunised with haemoglobin fraction (Hf). These animals showed a mean
10 level of protection from parasite challenge of 43.8%. This sera should contain antibodies reactive with haemoglobin and any other antigens present in the immunising fraction.

15 Ten plates with ca 2,000 pfu on each were used in the primary screening with a 1:500 dilution of pre-adsorbed sera. Thirty positive plaques were chosen and these were subjected to three or four further rounds of screening until all plaques on the plates were positive
20 indicating pure clones. Lysates of positive plaques were then prepared and the DNA analysed by PCR using λ forward and reverse primers. Of the thirty positives selected only twenty produced PCR products; the remaining ten were therefore disregarded. Clones were
25 classified into groups on the basis of the size of the PCR fragment (Figure 1).

	Group	Size of PCR fragment	Clones
	1	~1700bp	D6
30	2	~1600bp	B5&D5
	3	~1400bp	A1, A4, A5, B1, B4, B6, E3
	4	~1100bp	C4
	5	~1000bp	C2, D1, D7, E2
	6	~ 900bp	D8
35	7	~ 700bp	C1&D3
	8	~ 650bp	A8
	9	~ 550bp	E4

- 25 -

13. Sub cloning of phage inserts

Subcloning was performed with D6 of clone Group 1 (1700 bp) and B1 of Group 3 (1400 bp). The λ PCR products of these two clones were subcloned directly into the pGem[®]-T plasmid. White colonies were picked and screened by double digestion with Sac I and Apa I restriction enzymes. A clone with a 1600-1700 bp insert was isolated from D6 and a 1400-1500 bp insert was obtained from clone B1.

14. Sequence analysis of clone D6

DNA from the recombinant plasmids was sequenced commercially following purification using Wizard[™] λ preps. From clone D6 a partial sequence of ca 420 bases was obtained. The deduced 141 amino acid sequence was compared to sequences from available databases and was found to show significant homology with the C-terminal end of β -tubulins from various organisms. β -tubulins are proteins of 440-450 amino acids in length, corresponding to ca 1320 bases, therefore clone D6 of ca 1700 bases may contain the entire F.hepatica β tubulin gene. Figure 2 shows the alignment of the partial D6 sequence with β -tubulin from Toxoplasma gondii. In the region of overlap the D6 sequence shows 64% identity and 73% similarity with the C-terminus of the protozoan tubulin.

15. Sequence analysis of clone B1

Clone B insert was estimated to be ca 1400 bp in length by PCR amplification using λ primers. Approximately 1200 bases of the insert were sequenced in the 5' to 3' direction. This revealed a start codon ATG and an open reading frame of ca 580 bases ending with the in-frame termination codon TAG. Downstream from the termination

- 26 -

codon was stretch of about 20 adenine residues (Poly A tail), preceded by two poly adenylation sequences, AAAATAAA and AATA, indicating that the clone was complete at its 3' end. The DNA has a 5' untranslated region of ca 200 bases and a 3' untranslated region of ca 700 bases.

Clone B1 is predicted to encode a protein of 194 amino acids with a calculated molecular mass of 21,646 Da. When used to screen protein sequence databases, the predicted amino acid sequence shows a highly significant identity with a novel family of antioxidant proteins, the peroxiredoxin family. Alignment of clone B1 with rat thiol specific antioxidant (TSA, GenBank accession no. P35704), human natural killer cell enhancing factor B, (NKEF B, accession no. P31945), human proliferation associated gene, (PAG, accession no. X67951), human TSA (Lim *et al.*, 1994, accession no. P35701), and Onchocerca volvulus TSA (accession no. 009385) is shown in figure 3.

The protein with the highest identity is rat TSA; 57.0% and 74.6% similar. The other identities are as follows human NKEF B 56.9%, (71.5% similar) human PAG 53.8% (73.0% similar), human TSA 53.7% (71.0% similar) and Onchocerca volvulus TSA 2.0% (33.7% similar). Similarity was observed over the entire length of the sequences and two highly conserved domains were observed. The first of these is a sixteen amino acid stretch at ca positions 40-60, - F Y P L D F T F V C P T E I I A -. The second shorter domain - H G E V C P A - is found at ca positions 165-175.

16. Expression of fusion protein by clone B1

The plate wash supernatant method was used to make fusion proteins from clone B1 phage. The resulting

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supernatant and supernatant from E. coli infected with wild type phage were analysed on reducing SDS PAGE (Figure 4A). In the wild type preparation a protein with the same molecular mass as β -galactosidase was observed (lane 1). In B1 supernatants this protein was absent but a larger protein of molecular mass ca 160 kDa, not found in wild type, was observed (lane 2). To determine if this was the fusion protein, the gel was blotted onto nitrocellulose paper and probed with anti- β -galactosidase antibody (Figure 4B). Binding of the antibody to the large protein confirmed its identity as a β -galactosidase fusion protein (lane 2). The antibody also bound the wild type β -galactosidase molecule (lane 1) and a number of other proteins common to both supernatants.

17. Northern analysis

Primers designed from the conserved domains of the antioxidant proteins (around the VCP motifs at ca positions 50 & 170), were used to amplify a DNA fragment of ca 400 bp in length. This was ^{32}P labelled and used to probe both F.hepatica and bovine RNA, which were analysed on an agarose gel prior to blotting. A single transcript of ca 750 kb was found in the F.hepatica RNA (Figure 5 lane 1). No peroxiredoxin-similar binding was observed in the bovine RNA (Figure 5 lane 2).

18. Antioxidant activity of mature fluke extract

Antioxidant activity was measured as the ability of liver fluke extract to prevent the inactivation of glutamine synthetase by a mixed iron thiol inactivation system. Figure 6 shows the inactivation of glutamine synthetase by iron and DTT in the presence of various levels of liver fluke homogenate (LFH). Incubation of glutamine synthetase with iron and DTT results in a 70%

- 28 -

loss of the enzymes activity. The presence of LFH provides dose dependent protection, with 0.3 mg, 0.6 mg and 0.9 mg LFH restoring 50%, 61% and 75% glutamine synthetase activity, respectively.

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- 30 -

CLAIMS:

1. A vaccine composition for use in combating a parasitic infestation of helminths in a mammal wherein
5 the antigenic material comprises a peroxiredoxin and/or a β -tubulin molecule, in at least partially purified form, or an antigenic fragment or epitope, component, precursor, analogue, variant or functionally equivalent derivative thereof, together with a carrier and/or
10 adjuvant.
2. A vaccine as claimed in claim 1 wherein said antigenic material is derived from a Fasciola or Dicrocoelium species.
15
3. A vaccine as claimed in claim 1 or claim 2 wherein said antigenic material is derived from Fasciola hepatica.
- 20 4. A vaccine as claimed in any one of claims 1 to 3 wherein said antigenic molecules incorporated into said vaccine are at least 75% pure.
5. A vaccine as claimed in claim 4 wherein said
25 antigenic molecules incorporated into said vaccine are at least 95% pure.
6. A vaccine as claimed in any one of claims 1 to 5 further comprising one or more additional antigenic
30 determinants to form a polyvalent vaccine.
7. A vaccine as claimed in claim 6 wherein said additional antigenic determinants are Cathepsin L-type antigen and/or dipeptidyl peptidase antigen and/or a
35 haemoprotein antigen.

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8. The use of peroxiredoxin and/or β -tubulin molecules of fluke origin, in the preparation of a vaccine composition for combatting a parasitic infestation of helminths in a mammal.

5

9. A method of combating a parasitic infestation of helminths in a mammal comprising administering to said mammal a vaccine as defined in any one of claims 1 to 7 in an amount effective to combat said infestation.

10

10. A process for the preparation of a vaccine as defined in any one of claims 1 to 7 comprising bringing into association purified peroxiredoxin and/or β -tubulin molecules and one or more adjuvants or carriers.

15

11. A DNA molecule encoding a β -tubulin molecule or a peroxiredoxin molecule of the nucleic acid sequence shown in Fig. 2 or Fig. 4 or a fragment or homologue thereof.

20

12. A protein or polypeptide fragment thereof of the amino acid sequence shown in Fig. 2 or Fig. 4.

13. A vector comprising one or more nucleotide sequences as defined in claim 11.

25

14. A transformed host cell comprising the vector of claim 13.

15. A synthetic polypeptide comprising one or more amino acid sequences constituting a peroxiredoxin and/or a β -tubulin molecule or antigenic portions thereof, substantially corresponding to all or a portion of the amino acid sequences as shown in Figs. 2 and/or 4 or a functionally-equivalent variant thereof.

30

35

- 32 -

16. A process for the preparation of recombinant peroxiredoxin and/or β -tubulin polypeptides or antigenic fragments or epitopes thereof comprising culturing transformed host cells and isolating said peroxiredoxin and/or β -tubulin polypeptides or fragments or epitopes from the cultured cells.

17. A live or inactivated vaccine formulation, comprising an attenuated or virulent virus, or a host cell, having inserted therein a nucleic acid molecule as defined in claim 11, capable of stimulating an immune response against polypeptides encoded by the inserted nucleic acid molecule.

18. Monoclonal or polyclonal antibodies capable of inducing immunity to peroxiredoxin or β -tubulin molecules when administered to a mammal, said antibodies having an affinity for the variable region of one or more further antibodies, said further antibodies having an affinity for said peroxiredoxin or β -tubulin molecules.

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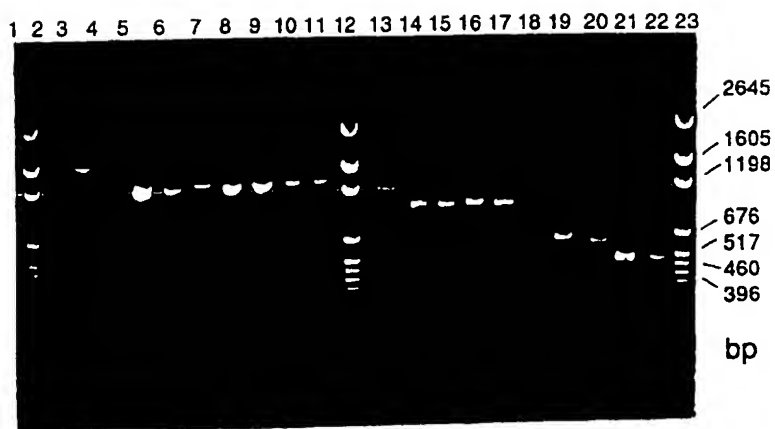
5

10

15

20

25



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30 Figure 1

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5	Z 1-50 clone D6 11 1-49	<div>10 20 30 40 50</div> CGGTNCCANT NCCCCNANCC CGGTANTTAA CCGGATTCCC ANANTGCCCC G ? ? ? ? ? P V ? N R I P ? ? P
	Z 51-100 clone D6 11 50-99	<div>60 70 80 90 100</div> AAAANGNCCC CCCCCNGAA TAAATTCCT NAANNMCNN GNTGGCCCAN Q ? ? P ? ? E O N S ? ? ? ? ? A ?
	Z 101-150 clone D6 11 100-149	<div>110 120 130 140 150</div> TTACCAACCC NNGAAACCA NAATNTGGG GNNCTNAGG GNNCCCAGAA L P T ? E T ? K ? G ? P ? ? P O N
10	Z 151-200 clone D6 11 150-199	<div>160 170 180 190 200</div> CTACACCAA NAATTTTNA ANCCAANAAA CNAANGCCCC CCTTTTGAAC ? H O ? I ? ? P ? N ? ? P P F E
	Z 201-250 clone D6 11 200-249	<div>210 220 230 240 250</div> CCACTCATGG GCGCTAACT TAAGGTGCC CCGCTGTTC GNGGTGAAT P T H G R L T O G G R P V P ? S N
15	Z 251-300 clone D6 11 250-299	<div>260 270 280 290 300</div> GTCCCATGAA ANAAGTGGAC GAACAGATGC TGAATGTGA GNAACAAGA V P J ? K W T H K C J M C ? N K E
	Z 301-350 clone D6 11 300-349	<div>310 320 330 340 350</div> ATTCCAAGCT ACTTGTGCA ATGGNATCCC GAATAACGTG AAAACTGCCG F O A T L S N ? I P N N V K T A
	Z 351-400 clone D6 11 350-399	<div>360 370 380 390 400</div> TTTGTGACAT TCCACCTAGG GCGCTTAAA TGTGGTCAC ATTGTGTGGC V C D I P P R G L K M S V T F V G
20	Z 401-450 clone D6 11 400-449	<div>410 420 430 440 450</div> AATAGTACTG CCATACAAGA ACTATTCAA CGTGTCTCG ACCAGTTCAC N S T A I O E L F K R V S E O F T
	Z 451-500 clone D6 11 450-499	<div>460 470 480 490 500</div> CGCAATGTC CGTCCAAAG CATTCTTGA TTGGTACACA GCGGAAGGTA A M F R R K A F L H W Y T G E G
25	Z 501-550 clone D6 11 500-549	<div>510 520 530 540 550</div> TGCACGAGAT GGAGTTCACC GAGGCCGAAT CGAACATGAA CGATCTGGTC H D E H E I T E A E S N M N D L V
	Z 551-600 clone D6 11 550-599	<div>560 570 580 590 600</div> AGTGAATATC AGCAATACCA AGANGCAACC GCTCAGGAGG AAGGCGAATT S E Y O O Y O ? A T A E E E G E F
	Z 601-650 clone D6 11 600-649	<div>610 620 630 640 650</div> CCAGCTGANC GCGCGCCTA CCATTACCAG TTGCTCTGCT GTCAATCCC Q L ? A G A T I T S W S G V K S
30	Z 651-700 clone D6 11 650-699	<div>660 670 680 690 700</div> ACCATGGCGC CGAGCATCG ACGNNGCCCA ATCGCCCTNN GTAGCGNTTA Q H G A G A S T ? P N R P ? B ? L

Figure 2

5	clone D6	1	-----
	8 Tubulin Toxoplasma	1	MREIVHVQGGQCGNQIGAKFWEVISDEHGIDPTG
	clone D6	1	-----
	8 Tubulin Toxoplasma	35	TYCGDSDLQLERINVVFYNEATGGRFVPRAILMDL
	clone D6	1	-----
	8 Tubulin Toxoplasma	69	EPGTMDSVRAGPFGQLFRPDNFVFGQTGAGNNWA
10	clone D6	1	-----
	8 Tubulin Toxoplasma	103	KQHYTEGAELIDSVLDVVRKEAECCDCLOGFQIT
	clone D6	1	-----
	8 Tubulin Toxoplasma	137	HSLGGGTGSGMCTLLISKVREEYPDRIMETFSVF
	clone D6	1	-----
	8 Tubulin Toxoplasma	171	PSPKVS DTVVEPYNATLSVHQLVENADEVQVIDN
	clone D6	1	-----
	8 Tubulin Toxoplasma	205	EALYDICFRTLKLTTP-YGDLNHLVSAAMSGVT
15	clone D6	1	-----
	8 Tubulin Toxoplasma	238	CCLRFP GQLNSDLRKLAVNLVFPFRLHFFLIGFA
	clone D6	1	-----
	8 Tubulin Toxoplasma	272	PLTSRGSQQYRALSVP ELTQQHFD AKNMHCASDP
	clone D6	1	-----RCJMCZKKEF-----QATLSN
	8 Tubulin Toxoplasma	306	RHGRYL TASAMPRGRMS TKEVDEQMLNVQNKNS
20	clone D6	18	---IPNNVKTAVCDIPRGLKMSVTFVGNSTAI
	8 Tubulin Toxoplasma	340	YFVEWIPNNMKSSVCDIPRGLKMSVTFVGNSTAI
	clone D6	48	QELFKRVSEQFTANFRKAFLLHWYTGEGMDEMEF
	8 Tubulin Toxoplasma	374	IQEMFKRVSDQFTAMFRKAFLLHWYTGEGMDEMEF
	clone D6	82	TEAESNMNDLVSEYQYQZATAEEEGEPOLZAGA
	8 Tubulin Toxoplasma	408	PTEAESNMNDLVSEYQYQZATAEEEGEPDEEEG
	clone D6	116	TITSWSGVKSQHGAGASTZPNRPZBZL
25	8 Tubulin Toxoplasma	442	EMGAEEGA-----

Figure 3

Nucleotide sequence and predicted amino acid sequence of clone B1
containing the peroxiredoxin gene

```

-178          TCGCTCACTATAGGGCGAATTGGGCCCCGACGTCGCAT
-141 GCCCCCGGCCGCCATGGCCGCGGGATTGGTGGCGACGACTCCTGGA
-95  GCCCGTNAGTATCAGCGGAATTCGGGTGTGATCGCAATCAGTGCTCTC
-47  CGGGGCGCCATCCACTTCCCCACTCTCATCCGCATTCCCAAAGACCG

1   ATG TTG CAG CCT AAT ATG CCC GCC CCG AAT TTT TCT GGA
    met leu gln pro asn met pro ala pro asn phe ser gly (12)
40  CAG GCG GTA GTG GGC AAG GAG TTC GAA ACC ATC AGT TTA
    gln ala val val gly lys glu phe glu thr ile ser leu (25)
79  TCA GAC TAC AAG GGC AAA TGG GTG ATT CTC GCC TTC TAT
    ser asp tyr lys gly lys trp val ile leu ala phe tyr (38)
118 CCA CTT GAT TTC ACG TTC GTG TGT CCA ACG GAA ATA ATC
    pro leu asp phe thr phe val cys pro thr glu ile ile (51)
157 GCG ATC AGT GAT CAG ATG GAG CAG TTC GCA CAA CGT AAC
    ala ile ser asp gln met glu gln phe ala gln arg asn (64)
196 TGC GCC GTC ATC TTC TGC TCT ACT GAC TCG GTT TAT TCG
    cys ala val ile phe cys ser thr asp ser val tyr ser (77)
235 CAT CTG CAA TGG ACC AAA ATG GAT CGT AAG GTT GGC GGT
    his leu gln thr thr lys met asp arg lys val gly (90)
274 ATA GGC CAG CTG AAC TTC CCG CTG CTG GCA GAC AAG AAT
    ile gly gln leu asn phe pro leu leu ala asp lys asn (103)
313 ATG TCT GTC TCT CGC GCC TTT GGT GTT CTG GAT GAG GAA
    met ser val ser arg ala phe gly val leu asp glu (116)
352 CAG GGT AAT ACC TAC CGT GGC AAT TTC CTC ATC GAT CCC
    gln gly asn thr thr arg gly asn phe leu ile asp pro (129)
391 AAG GGG GTC CTG CGC CAG ATC ACG GTG AAT GAC GAC CCG
    lys gly val leu arg gln ile thr val asn asp asp pro (142)
430 GTG GGC CGT TCC GTT GAA GAA GCC TTG CGT CTG CTC GAT
    val gly arg ser val glu glu ala leu arg leu leu asp (155)
469 GCA TTC ATA TTC CAC GAG GAG CAT GGA GAG GTC TGC CCG
    ala phe ile phe his glu glu his gly glu val cys pro (168)
508 GCG AAC TGG AAG CCT AAA AGC AAG ACC ATC GTG CCT ACT
    ala asn trp lys pro lys ser thr ile val pro thr (181)
547 CCG GAT GGA TCC AAA GCA TAT TTC TCC TCA GCC AAC TAG
    pro asp gly ser lys ala tyr phe ser ser ala asn *** (193)

586 TGAACAAGGGTGCTTAATCCCGGCTCTGTGTTTCGTTTCTGGTTTAAAA
635 TAAATTAGATAATACGGTGCAAAAAAAAAAAAAAAAAACGGAATNCCGGT
684 ACGGTAACAGTTCCCAAGCGCAACAGTATGATGAGAATCCAACGTGATTA
733 TCGTCTTGGAATCGCTCATTGGTTTCGCAACCAGTTTTCGACTGNAGGC
782 AACCGCATTCAAGGATTGTGGCTCGCAACTTGCCGAATTGATGAATGTG
831 ACTGTGAAACCATGTGANACTACTCTGTGTAGTGNGTNTCGNGGNGANA
880 ACGCCCAACTGGAAATCACTTCCCGAACAAAGGAAGTTGGCAAGTCTTG
929 AAAGCAGTCGGCCGTNCAATAGTCGGACGTGTTTCTGCCCATCCCCCT
978 GGATGACTA
  
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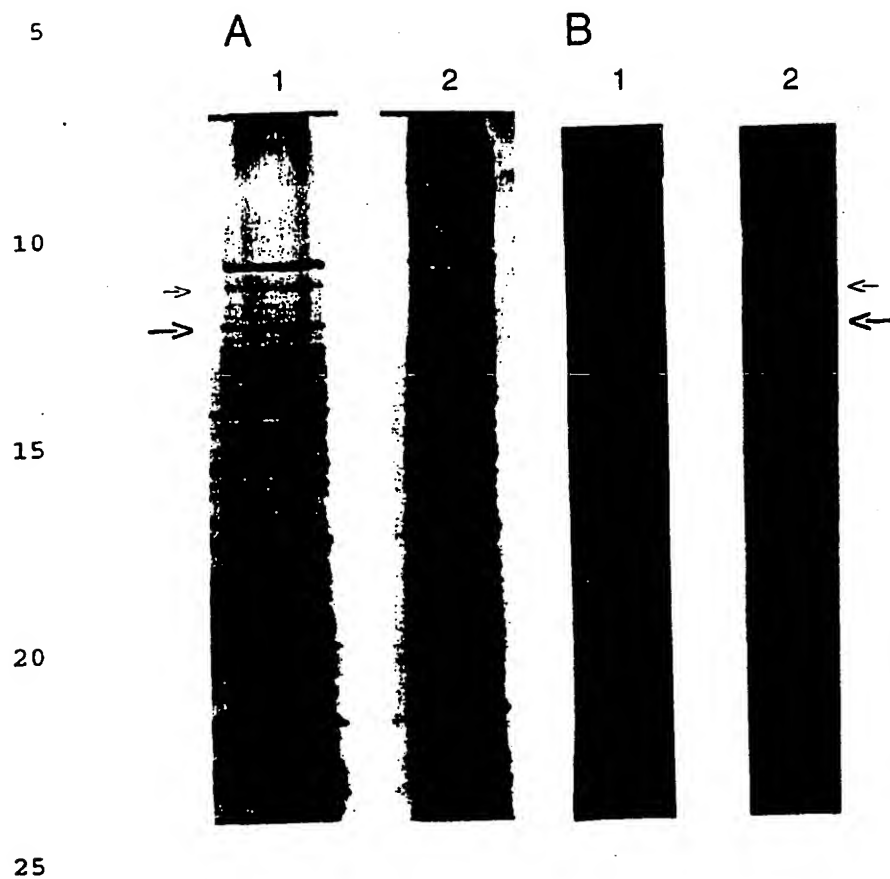
Figure 4

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clone B1	1	-----MLQPNMPAPNFSGOAVVGKEFE-TISLSDY
TSA rat	1	--MASGNAHIGKPA PDFTGTAVV-DGAFKEIKLSDY
NKFB human	1	--MASGNARIGKPA PDPKATAVV-DGAFKEVKLSDY
PAG human	1	--MSSGNARIGHAPNFKATAVMPDGQFKDISLSDY
TSA human	1	--MASGNARIGKPA PDPKATAVV-DGAFKEVKLSDY
TSA Oncocherca	1	EFKKRNVKLIGLSCDSADSHSRWADDILALYKMKCV
		↓
clone B1	30	KGKRWILAFYPLDFTFVCPTEIIAISDQHEQFAQRN
TSA rat	34	RGKYVVLFFYP LDFTFVCPTEIIAFSDHAEDFRKLG
NKFB human	34	KGKYVVLFFYP LDFTFVCPTEIIAFSDHAEDFRKL-
PAG human	35	KGKYVVLFFYP LDFTFVCPTEIIAFSDRAEEFKKLN
TSA human	34	KGKYVVLFFYP LDFTFVCPTEIIAFTTVKRTSAKLG
TSA Oncocherca	37	GCDSEKKLPYPIIADEDRSLATELGHMDFDERDEKG
		↓
clone B1	66	CAVIECSTDSVYSHLQWTKMDRKVGGIGOLNFPLLA
TSA rat	70	CEVLGVSVDSQFTHLAWINTPRKEGGLGPLNIPLLA
NKFB human	69	E-VLGVSVDSQFNHLAWINTPRKEGGLGPLNIPLLG
PAG human	71	COVIGASVDSHFCHLAWVNTPKKOGGLGPMNIPVLS
TSA human	70	CEVLGVSVDSQFTHLAWINTPRKEGGLGPLNIPLLA
TSA Oncocherca	73	NTLTARCVFIIGSDKTLKLSILYPATTGRNPFDEILR
		↓
clone B1	102	DKNMSVSRAFGVLDDEEQNTYRGNFLIDPKGVLRQI
TSA rat	106	DVTKSLSONYGV LKNDEGIA YRGLFIIDAKGVLRQI
NKFB human	104	DVTRRLSEYGV LKTDGIA YRGLFIIDGKGVLRQI
PAG human	107	DPKRTIAQDYGV LKADGIA YRGLFIIDGKILRQI
TSA human	106	DVTRRLSEYGV LKNDEGIA YRGLFIIDGKGVLRQI
TSA Oncocherca	109	VVDSLQILTAVKLVATPVQWKDGDCCVVLPTIIDNEA
		↓
clone B1	138	TVNDLPVGRSVDEALRLLD AFIH EEHGEVCPANWK
TSA rat	142	TVNDLPVGRSVDEALRLVQAFQYTOEHGEVCPAGWK
NKFB human	140	TVNDLPVGRSVDEALRLVQAFQYTOEHGEVCPAGWK
PAG human	143	TVNDLPVGRSVDETLRLVQAFQFTDXHGEVCPAGWK
TSA human	142	TVNDLPVGRSVDEALRLVQAFQYTOEHGEVCPANWK
TSA Oncocherca	145	KKLFGEKIHTIDLPSSGK-----
		↓
clone B1	174	PKSKTIIVPTPDGSKAYFSSAN
TSA rat	178	PGSDTIKPNVDGSKKEYFSKHNN
NKFB human	176	PGSDTIKPNVDGSKKEYFSKHNNNE
PAG human	179	PGSDTIKPNVDGSKKEYFSKQK
TSA human	178	PGRDTIKPNVDGSKKEYFSKHNN
TSA Oncocherca	0	-----

Figure 5

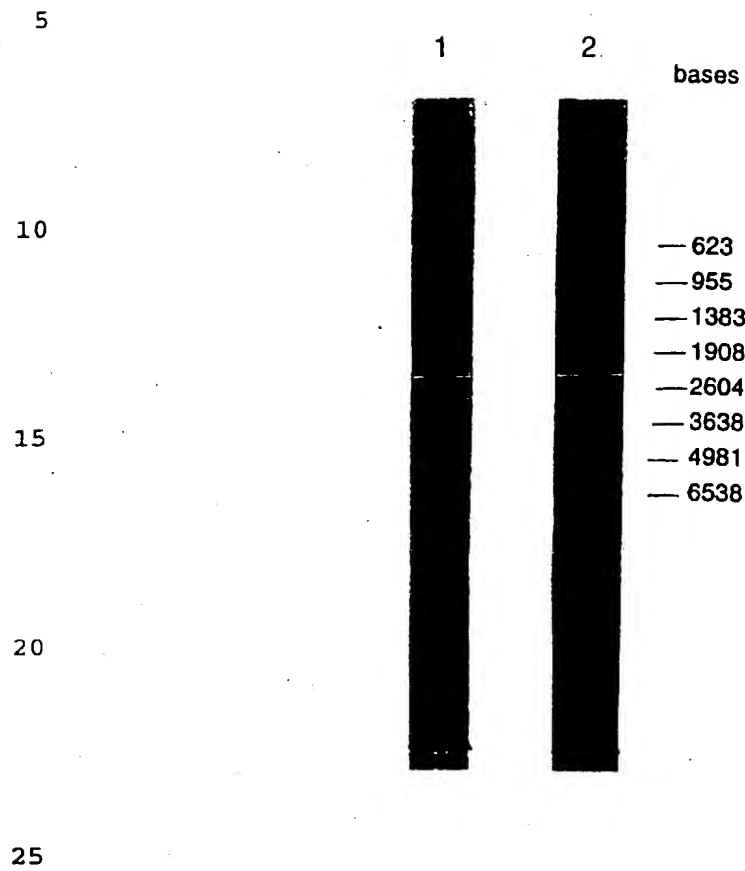
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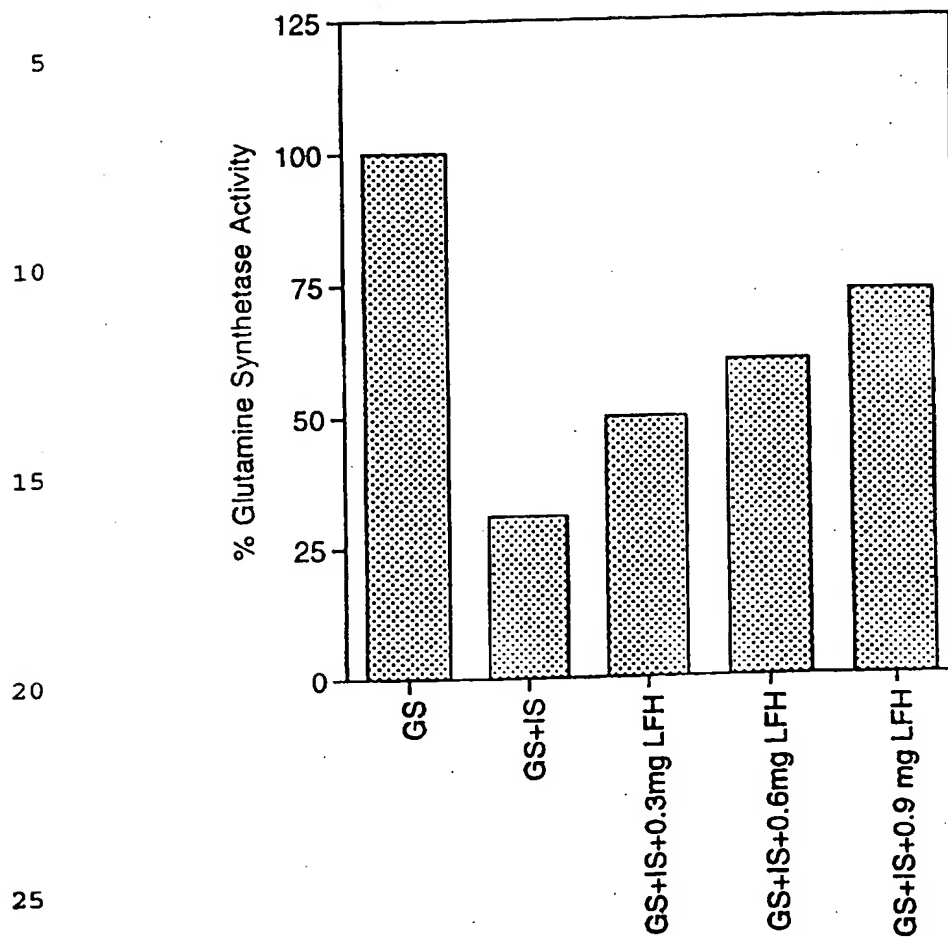
30 Figure 6

7/8

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30 Figure 7



30 Figure 8

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/12, C07K 14/435, 16/18, A61K 38/17, C12N 1/21 // (C12N 1/21, C12R 1:42)	A3	(11) International Publication Number: WO 97/47740 (43) International Publication Date: 18 December 1997 (18.12.97)
(21) International Application Number: PCT/GB97/01573 (22) International Filing Date: 11 June 1997 (11.06.97) (30) Priority Data: 9612214.8 11 June 1996 (11.06.96) GB (71) Applicant (for all designated States except US): MALLINCKRODT VETERINARY, INC. [US/US]; 421 East Hawley Street, Mundelein, IL 60060 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): DALTON, John, Pius [IE/IE]; Dublin City University, Dublin 9 (IE). ANDREWS, Stuart, John [GB/GB]; Mallinckrodt Veterinary Limited, Breakspear Road South, Harefield, Uxbridge, Middlesex UB9 6LS (GB). (74) Agent: FRANK B. DEHN & CO.; 179 Queen Victoria Street, London EV4 4EL (GB).	(81) Designated States: AU, BR, CA, CN, MX, NZ, RU, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 26 March 1998 (26.03.98)	
(54) Title: VACCINE CONTAINING A PEROXIREDOXIN AND/OR A β -TUBULIN (57) Abstract The present application relates to vaccine compositions comprising peroxiredoxin and/or β -tubulin antigenic material, preferably of <i>Fasciola</i> or <i>Dicrocoelium</i> origin, for use in combatting a parasitic infestation of helminths in a mammal. It further relates to nucleic acid sequences which encode peroxiredoxin and/or β -tubulin molecules and the amino-acid sequences thereof, vectors comprising said nucleic acid sequences and cells transformed with such vectors.		

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 97/01573

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C07K14/435 C07K16/18 A61K38/17 C12N1/21
 //(C12N1/21, C12R1:42)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 10583 A (MALLINCKRODT VETERINARY INC ;DALTON JOHN PIUS (IE); ANDREWS STUART) 11 April 1996 cited in the application see the whole document ---	1-18
A	WO 90 08819 A (DARATECH PTY LTD) 9 August 1990 cited in the application see the whole document ---	1-18
A	WO 94 09142 A (MATTHEWS DEREK PETER ;PITMAN MOORE INC (US); DALTON JOHN P (IE)) 28 April 1994 cited in the application see the whole document ---	1-18

-/-

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

5 November 1997

Date of mailing of the international search report

18.02.98

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Mandl, B

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/GB 97/01573

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 94 28925 A (MATTHEWS DEREK PETER ;MALLINCKRODT VETERINARY INC (US); DALTON JOH) 22 December 1994 cited in the application see the whole document	1-18
A	--- CHAE H. Z. ET AL.: "CLONING AND SEQUENCING OF THIOL-SPECIFIC ANTIOXIDANT FROM MAMMALIAN BRAIN: ALKYL HYDROPEROXIDE REDUCTASE AND THIOL-SPECIFIC ANTIOXIDANT DEFINE A LARGE FAMILY OF ANTIOXIDANT ENZYMES." PROC. NATL. ACID. SCI. USA, vol. 91, 1994, pages 7017-7021, XP002045065 see the whole document	11-16
A	--- EMBL database entry OV400; accession number R95400; 6. September 1995; Blaxter M. L. et al.: 'Genes expressed in Brugia malayi infective third stage larvae.' XP002045832 see abstract & BLAXTER M.L. ET AL.: "Genes expressed in Brugia malayi infective third stage larvae." MOL. BIOCHEM. PARASITOL., vol. 77, April 1996, pages 77-94, XP002045066 see page 88, left-hand column, last line - right-hand column, line 8 see page 90, right-hand column, line 5 - page 91, left-hand column, line 3	1-16
T	--- MCGONIGLE S. ET AL.: "Cloning of peroxiredoxin, a novel antioxidant enzyme, from the helminth parasite Fasciola hepatica." PARASITOLOGY, vol. 115, no. 1, July 1997, pages 101-104, XP002044922 see the whole document -----	1-5,8-16

INTERNATIONAL SEARCH REPORT

In ternational application No.

PCT/GB 97/ 01573

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim(s) 9
is(are) directed to a method of treatment of the human/animal
body, the search has been carried out and based on the alleged
effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Subject 1) Claims 1-18 partially
Subject 2) Claims 1-18 partially

See continuation sheet PCT/ISA/210 for further information.

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-18 partially (subject 1)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1-18, partially

Vaccine composition comprising helminth peroxiredoxin; use of helminth peroxiredoxin in the preparation of a vaccine; process for the preparation of a vaccine comprising helminth peroxiredoxin; peroxiredoxin of *Fasciola hepatica* and fragments thereof, gene encoding it, vectors containing said gene and host cells containing said vector, process for the preparation of helminth peroxiredoxin by said host cells; antiidiotypic antibodies of helminth peroxiredoxin; live or inactivated vaccine comprising a virus or a host cell presenting helminth peroxiredoxin.

2. Claims: 1-18, partially

Vaccine composition comprising helminth beta-tubulin; use of helminth beta-tubulin in the preparation of a vaccine; process for the preparation of a vaccine comprising helminth beta-tubulin; beta-tubulin of *Fasciola hepatica* and fragments thereof, gene encoding it, vectors containing said gene and host cells containing said vector, process for the preparation of helminth beta-tubulin from said host cells; antiidiotypic antibodies of helminth beta-tubulin; live or inactivated vaccine comprising a virus or a host cell presenting helminth beta-tubulin.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 97/01573

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